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**Thermolabile liposome with a controlled release temperature**

**Description**

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The invention relates to a thermolabile liposome with a controlled release temperature for the liposome content, in particular a liposome which is stable at 37°C in serum and with a controlled release temperature of between 40 and 80°C.

Liposomes are artificially formed vesicles consisting of lipid bilayers which enclose an aqueous compartment (Bangham et al., 1965). Originally also utilized as a model system for a cell membrane, liposomes have recently been developed further, especially for pharmaceutical transport. Liposomes can increase the tolerability of active compounds here (lowering of the active toxicity of amphotericin B by liposomal formulation (AmBisome®) by a factor of 75 (Proffitt et al., 1991)). However, they also increase the possibility of transporting pharmaceuticals specifically into diseased tissue (Forssen et al., 1992). After intravenous administration, liposomes are mainly absorbed in cells of the reticuloendothelial system (RES) of the liver and spleen (Gregoriadis and Nerunhun, 1974). In order to be able to utilize liposomes as pharmaceutical vehicles for cells outside the RES, it was attempted to increase the circulation time of the liposomes in the blood. Especially in tumors, which are often very highly vascularized (Jain, 1996) and whose vessels are particularly permeable due to dilated interendothelial connections, a large number of fenestrations, and discontinuous basal membranes (Murray and Carmichael, 1995), the probability of absorption of liposomes would be massively increased thereby.

A first problem in the use of liposomes for the

transport of active compounds or labelling substances in body fluids therefore lies in the increase in the circulation time in the serum. Indeed, it has already been found that due to covalent bonding of methoxy-  
5 polyethylene glycols to the liposomal membrane the premature recognition of the liposomes by the RES is prevented and thus the circulation time of the liposomes can be improved. In addition to an improvement in the circulation time, however, there is  
10 also great interest in a possibility of achieving a controlled release of the liposome ingredients at a certain temperature by means of the action of temperature.

15 The invention is therefore based on the object of making available a liposome which has a significantly improved half-life in the serum, compared with the customary half-life of known liposomes of the order of magnitude of around 4 hours, and which is constituted  
20 such that the content of the liposomes is rapidly released at a certain temperature.

This object is achieved according to the present invention by means of a liposome with a controlled  
25 release temperature for the liposome content, which is characterized in that it is essentially formed from at least one phosphatidylcholine with a main transition temperature in the range from 0 to 80°C and more than 15 to 70% by weight of phosphatidyloligoglycerol.  
30 According to an older proposal, it was only possible to obtain liposomes having a maximum phosphatidyloligoglycerol content of 15% by weight. Now, however, it has surprisingly been found that it is possible to increase the phosphatidyloligoglycerol content up to  
35 70%, so that the range of the achievable release temperatures of the liposomes is extended even more, but especially the half-lives are again improved.

According to a preferred embodiment, the liposomes according to the invention additionally contain smaller amounts of alkylphosphocholines, preferably 10 to 15% by weight. Suitable substances are, for example, 5 hexadecylphosphocholine, oleylphosphocholine and ether lysolecithins. In the ether lysolecithins, the hydroxyl group in position 2 of the glycerol can be methylated or free. In this embodiment, it is possible to increase the release of the substances enclosed in the liposome from approximately 70% without increasing the content 10 of alkylphosphocholine to virtually 100%, which is to be attributed to an acceleration of liposome opening. In addition, the alkylphosphocholines have an antitumor effect due to temperature-dependent release from the 15 liposomes.

Liposomes constructed according to the invention have significantly improved half-lives of up to more than 25 hours in the serum and the content(s) can be rapidly 20 and completely released at a predetermined temperature by suitable choice of the components and amounts of the components as a function of their main transition temperature.

25 Preferably, the liposome according to the invention is composed of approximately 20 to 75% by weight of dipalmitoyllecithin (1,2-dipalmitoylglycero-3-phosphocholine), approximately 10 to 25% by weight of distearoyllecithin (1,2-distearoylglycero-3-phospho- 30 choline) and more than 15 to approximately 50% by weight of dipalmitoylphosphoglyceroglycerol. Such a preferred composition is stable at 37°C in the serum, but rapidly releases the content on exceeding a temperature of 40°C.

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A further preferred composition with an improved release of the substances enclosed in the liposome consists of approximately 15 to 70% by weight of

dipalmitoyllecithin, approximately 10 to 25% by weight of distearoyllecithin and more than 15 to approximately 45% by weight of dipalmitoylphosphoglyceroglycerol.

- 5 The abovementioned preferred composition of the liposome according to the invention can be tailor-made for other temperature ranges by choice of components with the main transition temperature suitable in each case. In table 1, the main transition temperatures ( $T_M$ )
- 10 of phosphatidylcholines whose main transition temperatures lie in the range from 0 to 80°C are indicated. The main transition temperatures are, as can be seen from the table, dependent on the chain length and the distribution on positions 1 and 2 of glycerol-
- 15 3-phosphocholine or on positions 1 and 3 of glycerol-2-phosphocholine.

Table 1

| T <sub>M</sub> | Phosphatidylcholine      |
|----------------|--------------------------|
| 5°C            | 1-palmitoyl-2-oleoyl-    |
| 7°C            | 1-stearoyl-2-oleoyl-     |
| 11°C           | 1-palmitoyl-2-lauroyl-   |
| 14°C           | 1-behenoyl-2-oleoyl-     |
| 17°C           | 1-stearoyl-2-lauroyl-    |
| 19°C           | 1,3-dimyristoyl-         |
| 23°C           | 1,2-dimyristoyl-         |
| 27°C           | 1-palmitoyl-2-myristoyl- |
| 33°C           | 1-stearoyl-2-myristoyl-  |
| 37°C           | 1-myristoyl-2-palmitoyl- |
| 39°C           | 1,3-dipalmitoyl-         |
| 41°C           | 1,2-dipalmitoyl-         |
| 42°C           | 1-myristoyl-2-stearoyl-  |
| 46°C           | 1-stearoyl-3-myristoyl-  |
| 48°C           | 1-stearoyl-2-palmitoyl-  |
| 52°C           | 1-palmitoyl-2-stearoyl-  |
| 53°C           | 1,3-distearoyl-          |
| 56°C           | 1,2-distearoyl-          |
| 66°C           | 1,2-diarachinoyl-        |
| 75°C           | 1,2-dibehenoyl-          |
| 80°C           | 1,2-dilignoceroyl-       |

- 5 The values presented in table 1 show that virtually any desired temperature in the indicated range from 0 to 80°C can be adjusted by use of fatty acids with an uneven chain length and suitable distribution on the glycerol parent structure.

The content of phosphatidyloligoglycerols in the liposome according to the invention is essential for the long circulation time in the serum which is necessary. Phosphatidyloligoglycerols and their  
5 preparation are disclosed in DE 196 22 224. Preferably, dipalmitoylphosphoglyceroglycerol (DPPG2) is used.

The thermolabile liposomes according to the invention are outstandingly suitable for use in various fields,  
10 but in particular in regional deep hyperthermia. Regional deep hyperthermia, which is used in combination with systemic chemotherapy in specialized clinical centers, presents itself as an ideal technique for tumor-specific liposomal transport and the  
15 subsequent release of a pharmaceutical from the liposomal shell. Thus, hyperthermia, on the one hand, promotes the extravasation of liposomes from tumor capillaries into the interstitium (Gaber et al., 1996). On the other hand, a release of the pharmaceutical from  
20 special thermosensitive liposomes can be induced by heating (Magin and Niesman, 1984). Additionally, there are numerous indications of an increased cytotoxic effect of cytostatics (Hahn et al., 1975), and of an immunomodulation (activation of NK cells; Multhoff et  
25 al., 1999) by regional deep hyperthermia.

The thermolability of the liposomes according to the invention is caused by the phase transition of the phospholipids within the liposome membrane. If the  
30 phase transition temperature is passed through, a short-term membrane instability and subsequent release of the liposomal content occur.

In the abovementioned regional hyperthermia, the tumor  
35 is specifically overheated regionally, so that the temperature rises above the threshold temperature for the release of the liposome content. Possible liposome contents here are in particular active compounds which

can be used in oncology, such as, for example, cytostatics. However, contrast agents, for example gadolinium, e.g. Magnevist®, Multihance® or Omniscan®, carboxyfluorescein, iodine-containing contrast agents  
5 which are derived from pyridines or aromatic carboxylic acids, or the like on their own or together with an active compound can also be released. The temperature-dependent release of gadolinium from the liposomes can be shown with the aid of MRT by means of a modified T1  
10 time (0.2 or 1.5 Teslar respectively). By use of contrast agents, such as gadolinium, noninvasive thermometry is made possible in which the temperature reached can be determined by MRC, which measures the gadolinium released. In this use of the liposomes  
15 according to the invention, a hyperthermia apparatus coupled with an MRC apparatus is expediently used. Use of liposomes with iodine-containing contrast agents for demonstration in computer tomography (for example for the thermoablation of liver metastases) is also  
20 conceivable.

A further type of use for the liposomes according to the invention is found in ophthalmology. On encapsulation of a fluorescent labeling substance, it  
25 can be demonstrated where the desired overheating has actually occurred, for example, in a laser treatment by release of the fluorescent active compound, such as, for example, carboxyfluorescein.

30 Analogously to the illustrated possibility of use in the eye, liposomes according to the invention can therefore be generally used for the purpose of making temperatures reached additionally determinable, e.g. if certain heating temperatures or the like are to be  
35 ascertained.

The liposomes according to the invention consist essentially of the substances indicated above, which

are preferably present in pure form. Impurities should be kept as low as possible, in particular a cholesterol content which is as low as possible should be present. Liposomes which are completely free of cholesterol are preferred, since cholesterol leads to a spreading of the phase transition temperature and thus to a thermal transition range which is too broad.

The thermolabile liposomes according to the invention are prepared in the customary manner by dissolving the lipids, e.g. in chloroform or chloroform/water/isopropanol, stripping off the solvent, expediently in vacuo in a rotary evaporator, and temperature-controlling the lipids with aqueous solutions of the ingredients to be encapsulated at temperatures which lie above the phase transition temperature. The duration of this temperature treatment is expediently 30 to 60 minutes, but can also be shorter or longer. By means of freezing-thawing processes which are repeated a number of times, for example freezing and thawing again 2 to 5 times, homogenization takes place. Finally, the lipid suspension obtained is extruded through a membrane of defined pore size at a temperature above the phase transition temperature in order to achieve the desired liposome size. Suitable membranes are, for example, polycarbonate membranes of defined pore size, such as 100 to 200 nm. Finally, nonencapsulated ingredient can optionally be separated off, for example by column chromatography or the like.

The following figures and examples illustrate the invention further.

Figure 1 shows the obtained values of the in vitro CF release from thermolabile liposomes.

*Liposome composition:*

DPPG:DSOC:DPPG2 = 3:2:5

Great stability in the presence of serum at 37 C (CF

release after 18 hours < 7%).

Figure 2 shows the influence of the release temperature of DDPG<sub>2</sub>/DSPC/DPPC liposomes by variation of the proportion of DSPC at the expense of DPPC.

Figure 3 shows the improvement in the CF release from DDPG<sub>2</sub>/DSPC/DPPC liposomes by increasing the proportion of DPPG<sub>2</sub> at the expense of DPPC (constant proportion of DSPC at 20%).

Figure 4 shows the photon correlation spectroscopy (PCS) of liposomes consisting of 30% by weight of DPPG<sub>2</sub>, 20% by weight of DSPC and 50% by weight of DPPC (mean size: 175 nm).

Example 1

a) The liposomes presented in table 2 are prepared in the manner described above.

5

Table 2

|    |                       |          |          |          |
|----|-----------------------|----------|----------|----------|
|    | DPPG <sub>2</sub> 30% | DSPC 0%  | DPPC 70% |          |
|    | DPPG <sub>2</sub> 30% | DSPC 10% | DPPC 60% |          |
| 10 | DPPG <sub>2</sub> 30% | DSPC 20% | DPPC 50% |          |
|    | DPPG <sub>2</sub> 30% | DSPC 30% | DPPC 40% |          |
|    | DPPG <sub>2</sub> 10% | DSPC 0%  | DPPC 90% |          |
|    | DPPG <sub>2</sub> 10% | DSPC 10% | DPPC 80% |          |
| 15 | DPPG <sub>2</sub> 10% | DSPC 20% | DPPC 70% |          |
|    | DPPG <sub>2</sub> 10% | DSPC 30% | DPPC 60% |          |
|    | DPPG <sub>2</sub> 0%  | DSPC 20% | DPPC 80% |          |
|    | DPPG <sub>2</sub> 10% | DSPC 20% | DPPC 70% |          |
| 20 | DPPG <sub>2</sub> 20% | DSPC 20% | DPPC 60% |          |
|    | DPPG <sub>2</sub> 30% | DSPC 20% | DPPC 50% |          |
|    | DPPG <sub>2</sub> 40% | DSPC 20% | DPPC 40% |          |
|    | DPPG <sub>2</sub> 50% | DSPC 20% | DPPC 30% |          |
|    | DPPG <sub>2</sub> 80% | DSPC 20% | DPPC 0%  |          |
| 25 |                       |          |          |          |
|    | DSPG <sub>2</sub> 10% |          | DPPC 90% |          |
|    | DSPG <sub>2</sub> 20% |          | DPPC 80% |          |
|    | DSPG <sub>2</sub> 30% |          | DPPC 70% |          |
| 30 | DSPG <sub>3</sub> 10% |          | DPPC 90% |          |
|    | DSPG <sub>3</sub> 20% |          | DPPC 80% |          |
|    | DPPG <sub>2</sub> 30% | DSPC 20% | DPPC 40% | 1PPC 10% |
|    | DPPG <sub>2</sub> 30% | DSPC 20% | DPPC 30% | 1PPC 20% |
| 35 |                       |          |          |          |
|    | DSPG <sub>2</sub> 20% |          | DPPC 70% | 1SPC 10% |
|    | DSPG <sub>2</sub> 20% |          | DPPC 60% | 1SPC 20% |

|   |                       |          |                               |
|---|-----------------------|----------|-------------------------------|
|   | DSPG <sub>2</sub> 20% | DPPC 70% | hexadecyl-PC 10%              |
|   | DSPG <sub>2</sub> 20% | DPPC 60% | hexadecyl-PC 20%              |
|   | DSPG <sub>2</sub> 20% | DPPC 70% | octadecyl-PC 10%              |
| 5 | DSPG <sub>2</sub> 20% | DPPC 60% | octadecyl-PC 20%              |
|   | DSPG <sub>2</sub> 10% | DPPC 80% | Et-18 OCH <sub>3</sub> PC 10% |
|   | DSPG <sub>2</sub> 10% | DPPC 70% | Et-18 OCH <sub>3</sub> PC 20% |
|   | DSPG <sub>2</sub> 10% | DPPC 60% | Et-18 OCH <sub>3</sub> PC 30% |

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#### Abbreviations:

|    |                             |  |
|----|-----------------------------|--|
|    | DDPC =                      | 1,2-dipalmitoyl-sn-glycero-3-phosphocholine  |
|    | DSPC =                      | 1,2-distearoyl-sn-glycero-3-phosphocholine   |
|    | DPPG <sub>2</sub> =         | 1,2-dipalmitoyl-sn-glycero-3-phospho-        |
| 15 |                             | diglycerol                                   |
|    | DSPG <sub>2</sub> =         | 1,2-distearoyl-sn-glycero-3-phospho-         |
|    |                             | diglycerol                                   |
|    | DSPG <sub>3</sub> =         | 1,2-distearoyl-sn-glycero-3-phospho-         |
|    |                             | triglycerol                                  |
| 20 | 1PPC =                      | 1-palmitoyl-sn-glycero-3-phosphocholine      |
|    | 1SPC =                      | 1-stearoyl-sn-glycero-3-phosphocholine       |
|    | Et-18 OCH <sub>3</sub> PC = | 1-octadecyl-2-methylglycero-3-phosphocholine |

25 They contain encapsulated carboxyfluorescein. Free carboxyfluorescein was separated off beforehand by column chromatography using Sephadex G75.

#### b) Chamber model:

30 The Syrian hamster chamber model (A-Mel-3 melanoma of the Syrian hamster) is suitable for the intravital microscopic detection of the carboxyfluorescein (CF) release from thermolabile liposomes in the hyperthermia field. In this, a transparent, dorsal skin chamber is implanted in a Syrian golden hamster. After  
35 implantation of the skin chamber, the implantation of cells of the hamster A-Mel-3 melanoma takes place on the subcutaneous tissue located in the chamber. Within a few days, a tumor several millimeters in size grows

within the dorsal skin of the hamster. The microcirculation and the fluorescence enrichment within the tumor can be observed using a modified vital microscope. The animals are additionally given a  
5 central venous catheter. With the aid of a heat exchanger located under the skin chamber, heating of the tumor to 42°C can be achieved locally. The tumor temperature can be measured directly with the aid of a temperature probe (Endrich, 1988).

10

In addition to vital microscopy, the process of MRT measurement in the chamber model is also established (Pahernik et al., 1999). In this, MRT images can be recorded analogously to microscopy.

15

The obtained values of the in vitro CF release are shown in figure 1. Furthermore, the influence of the release temperature of DPPG<sub>2</sub>/DSPC/DPPC liposomes by variation of the proportion of DSPC at the expense of  
20 DPPC is shown in figure 2. The improvement in the CF release from DPPG<sub>2</sub>/DSPC/DPPC liposomes by increasing the proportion of DPPG<sub>2</sub> at the expense of DPPC (constant proportion of DSPC at 20%) is shown in figure 3. Moreover, photon correlation spectroscopy of  
25 DPPG<sub>2</sub>/DSPC/DPPC liposomes is shown in figure 4.